BIOLOGIJA. 2004. Nr. 3. P. 21–26

A study of genetic variability and adaptation in wild *Rubus idaeus* L. using molecular markers

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Adaptive variability was studied in twenty accessions of *Rubus idaeus* L. by RAPD (random amplified polymorphic DNA) and SOD (superoxide dismutase) isozyme polymorphism methods. Thirty-six informative RAPD primers yielded 284 reproducible DNA bands; 81.05% of identified RAPD loci were polymorphic. The distribution of 230 polymorphic loci in the plants studied was compared with soil properties of their original habitats by the method of principal components in factor analysis. A significant correlation (1%) was found only with soil acidity (–0.65). Differences in SOD isozyme patterns were found in leaf extracts of the plants. Six main SOD isozyme profiles were distinguished. The evaluation of SOD polymorphism in *R. idaeus* accessions showed no clear adaptive dependence from the soil properties analyzed in this study.

Key words: molecular markers, RAPD, SOD, adaptive variability, *Rubus idaeus*

INTRODUCTION

The study of adaptive variation is one of the most important objectives for conservation of plant genetic resources [1]. The genetics of adaptation has been the subject of intensive study since the theory of natural selection was proposed [2]. But the details of the genetic basis of adaptive variation in natural populations are still largely unknown. It is discussed whether few genes with large effects or many genes with small effects underlie genetic variation in adaptive traits [3]. The development of molecular marker technologies gave a new impulse for more detailed studies of plant genomes. RAPD and isozyme are among the most popular types of molecular markers. RAPD markers have been demonstrated to be useful for the studies of taxonomic identities, systematic relationships, population genetic structure, species hybridisation, and parentage identification [4, 5]. Usually RAPDs show much higher values of diversity than isozymes. It is accepted that most RAPD loci consist of noncoding sequences and are pure indicators of adaptive traits [6–8]. On the other hand, some authors pointed out that the RAPD technique provides a powerful tool for obtaining loci of both coding and non-coding sequences [4, 9, 10]. They offer evidence that RAPD polymorphisms are at least partly adaptive and are determined by natural diversifying selection [4, 11–14]. This point of view is also supported by the fact that RAPD diversity patterns in natural populations are similar to those of the allozymic differentiation [4, 12, 13, 15]. Marker-assisted selection and cloning also demonstrate the potential of neutral molecular markers to track the adaptive variation [16].

The problem of RAPD-based adaptive variation was studied in various plant species: *Pinus sylvestris* [8], *Hordeum spontaneum* [7, 12], *Phytolacca dodecandra* [14], *Triticum dicoccoides* [4]. The spatially dependent genetic variation was established by RAPDs within and among red raspberry (*Rubus idaeus* L.) populations [17].

The objectives of our study were: 1) to estimate RAPD diversity in the group of arbitrarily selected accessions of *R. idaeus*, which have grown in habitats with different soil characteristics; 2) to study a possible correlation between the variability of mole-

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cular markers (RAPDs, SOD) and the ecological factors.

MATERIALS AND METHODS

Plant material. Clonal samples of *R. idaeus* along with soil samples were collected in the wild during the growth season in 2001. Each clonal sample contained three separated 1- or 2-cane plants. The samples were planted into the field collection of the Botanical Garden of Vilnius University and managed according to common horticular practices. The habitat characteristics of the accessions studied are shown in Table 1. Soil samples for acidity (pH) assessment were taken from the rhizosphere during plant digging: one 300 cm^3 cardboard box was filled in with soil without litter, stones and other residuals for each raspberry clone sample (accession – JL01, JL02, etc.). Then the soil samples were dried and analysed at the Chemical Analyses Laboratory of the Institute of Botany for acidity, percentage of nitrogen and humus as well as available phosphorus and potassium. Soil pH was established by the potentiometric method. Available phosphorus was detected by the photometric method after extraction of soluble phosphates with 0.2 M HCl solution. Available potassium was measured by flame photometry after extraction of soluble potassium contents with 0.2 M HCl solution. To find the concentration of total nitrogen, the soil samples were processed with boiling concentrated sulphur acid. Then the formed ammonium ions were detected by the photometric method, using sodium salicylate and hypochlorite solutions in alkaline medium. Humus was established by the oxidation method using potassium bichromate and sulphur acid solution [18].

DNA extraction and PCR amplification. RAPD analysis was performed using DNA from 20 different raspberry plants. DNA was purified from fresh young leaves (100 μg) ground to a powder in mortars under liquid nitrogen. A #K0512 genomic DNA purification kit (MBI Fermentas) was used for DNA extraction. Each amplification was performed in 25 μl containing 1 × PCR buffer (MBI Fermentas), 3.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM of the primer, 1.0 U of Taq DNA polymerase (MBI, Fermentas) and 50 ng of *R. idaeus* DNA. Thirty-six primers 10 bp in length were selected from 44 (Roth Random Primer kits 270, 380, 470 and 14 primers from MBI Fermentas). The RAPD reactions were

Table 1. **Properties of original habitats of studied *Rubus idaeus* L. accessions**

DNA number	Collecting number	Habitat description	Characteristics of the soil of original site				
			Acidity (pH_{KCl})	Humus content, %	Total nitrogen content, %	Available P_2O_5, mg/kg	Available K_2O, mg/kg
1	JL01010425	Narrow belt between lakeside and roadside	7.12	6.20	0.48	123.30	130.70
2	JL02010425	Cutting of sprucewood	4.12	5.90	0.23	106.70	112.60
3	JL04010427	Alderwood glade	6.14	5.75	0.37	116.30	230.10
4	JL05010427	Oakwood glade	7.02	7.48	0.42	59.20	132.20
5	JL22010711	Cutting of sprucewood	3.56	4.32	0.20	24.60	53.80
6	JL36010908	Cutting of mixed forest on peat	4.01	28.52	1.93	90.10	708.20
7	JL45010926	Margin of broadleaved forest	4.85	1.80	0.13	145.50	103.00
8	JL18010625	Sandy lagoon coast	4.75	0.97	0.02	65.70	28.50
9	JL52010930	Cutting of pinewood, on peat	3.42	24.74	1.10	158.80	428.20
10	SS01011014	Peatland	6.78	9.57	0.73	386.90	416.00
11	JL03010425	Mature pinewood	5.46	3.94	0.26	95.00	254.10
12	JL06010513	Lakeside, mixed forest	5.48	3.33	0.22	64.60	136.80
13	JL08010523	Cutting of mixed forest	5.62	8.12	0.58	252.50	101.60
14	JL09010523	Field margin, river slope	6.60	4.58	0.22	129.20	260.80
15	JL10010527	Cutting of mixed forest	4.01	5.06	0.28	59.60	80.20
16	JL11010530	Electric power line, mixed forest	5.15	7.48	0.48	25.40	53.00
17	JL12010605	Electric power line, broadleaved forest	5.97	2.78	0.24	68.60	67.90
18	JL14010607	Peatland	4.75	28.96	3.06	161.00	238.60
19	JL15010609	Pinewood	4.02	1.80	0.04	130.50	22.30
20	JL16010610	Narrow belt between fen and dry sprucewood	3.05	5.28	0.13	17.80	55.40

carried out in Thermo Cycler (Biometra) under conditions described earlier [19].

The amplification products were analysed by electrophoresis in 1.6% TBE agarose gel (MBI Fermentas), stained with ethydium bromide and photographed using a BioDocAnalyse system (Biometra). All reactions were repeated at least twice. Only clear and reproducible DNA bands were scored.

Superoxide dismutase (SOD, EC 1. 15. 1. 1. 1) extraction and assay. Leaf discs of 20 plants were homogenised in pre-colded extraction buffer consisting of 50 mM potassium buffer, pH 7.8 (1 g plant fresh weight : 1 ml buffer). The homogenate was centrifuged at 12 500 rpm for 15 min at 4 °C. The supernatant was used on the same day for electrophoretic analysis. The native polyacrylamide gel electrophoresis was performed at 4 °C using a 4% stacking gel and 9% separating gel (200 V, 40 mA) using Tris-glycine buffer (pH 8.3) [20]. Twenty microlitres of crude extract from leaves was loaded in each line. SOD isozymes were localised on the gels by the method of NBT reduction. After electrophoresis the gels were incubated with a staining solution for an hour at 37 °C in the dark [21].

Data analysis. DNA bands were scored as present (1) or absent (0) using BioDocAnalyse software (Biometra). Bands of identical size amplified with the same primer were considered to be the same locus consisting of two alleles. A pairwise comparison of banding patterns was evaluated using the TREECON program for Windows [22]. The genetic distance was calculated according to formula described by Nei and Li [23]. The dendrogram was constructed by applying the UPGMA clustering method and the Nei and Li distance matrix. Only polymorphic fragments were used for evaluation of data (SAS V8 program package). The method of principal components in factor analysis was applied to analyse the data from RAPD fingerprints by the FACTOR procedure [24]. The Pearson correlation and its p-value were calculated to relate the factor analysis results to the soil characteristics (CORR procedure). The STEPDISC SAS procedure was applied to select RAPDs contributing most to the differentiation of individuals grouped by soil acidity properties. The least significance level 0.01 was used for the selection of single RAPD.

RESULTS AND DISCUSSION

To reveal a possible correlation between local adaptation of *R. idaeus* plants and genotype-specific RAPD patterns, we performed a detailed analysis of the genomes of selected plants. In our study, twenty accessions of *R. idaeus* were analysed using 44 RAPD primers; 36 of them yielded informative, polymorphic products. A total of 284 reproducible bands were obtained; 81.05% of the identified loci were polymorphic. The RAPD primers amplified 5 to 13 scorable DNA bands per genotype. The size of amplification products ranged from 190 bp to 3275 bp. The values of the genetic distance GDxy were calculated for pairwise comparisons of these 20 genotypes. The genetic distances for all 190 pairs ranged from 0.176 to 0.318. The UPGMA dendrogram constructed from the GDxy values shows the level of divergence among studied accessions (Fig. 1). The pattern of clustering was mainly geographically independent. For example, the first largest cluster includes eight individuals sampled from a territory that covers a great part of Lithuania. This suggests that geographic distance alone is not enough to explain genetic divergence. The clustering also can be influenced by the peculiarities in ecological conditions. Thus, the next step in our work was to test this hypothesis, assessing a possible correlation between RAPD patterns and soil properties at sites of origin of the accessions studied. The chemical properties of soil are very important for plant growth and survival. Habitat heterogeneity, combined with natural selection, often results in multiple, genetically distinct ecotypes within a single species [25]. In our study, we evaluate the possible local adaptation of different *R. idaeus* plants origi-

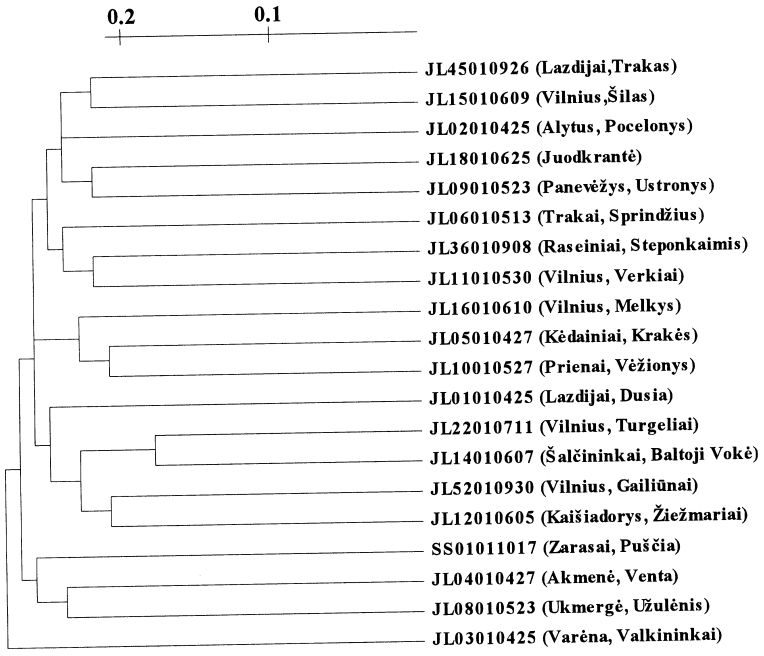

Fig. 1. UPGMA dendrogram of 20 raspberry accessions based on the genetic distance (Gdxy) values from RAPD data

nated from sites with different soil characteristics (Table 1). Twenty raspberry samples were obtained from the collection of Botanical Garden of Vilnius University, where they had been grown under even ecological conditions at least for two years. The principal component method in factor analysis was applied to analyse data from RAPD fingerprints to detect a possible correlation between RAPD markers and soil properties. The distribution of 230 polymorphic RAPD loci among 20 accessions was compared with the following soil variables: nitrogen, phosphorus, potassium, humus content and soil acidity (Table 2). A significant correlation (1%) was found only with soil acidity (–0.65). The curvilinearity test revealed the significance of the linear model (Fig. 2.) About 22 markers scored from 230 RAPD bands using 36 primers were mainly responsible for the correlation between DNA polymorphism and soil acidity. After applying the STEPDISC procedure, stepwise selection revealed 9 RAPDs contributing most to the separation of groups of individuals by soil acidity properties: 380b3-1550, MP4-1015, A4-530, A4-470, 470b7-800, 380b3-1390, B6-1000, A7-730, MP2-605.

The first three principal components from principal component analysis explained a total of 64.5% of the variation among samples. Principal component analysis (PCO) showed genetic differences among the individuals regarding soil acidity of their original habitats. The PCO plot indicates some slope in spatial grouping of accessions starting from the location of samples possibly adapted to acidic soils towards samples originally grown in soil with a higher pH (Fig. 3).

These observations are consistent with previous results obtained with different DNA markers for different collections [26, 27]. Our results also suggest that the non-coding part of the raspberry genome is subject to natural selection.

Isozymes are another class of genetic markers widely used in plant studies. There are many studies showing the role of isozymes as markers of adaptive traits [13, 28–31]. Drought, nitrogen deficiency, low temperatures and soil acidity are the most important abiotic stress factors for plants. All these factors induce oxidative stress in plant cells.

Table 2. **Correlation of factor analysis estimates with soil characteristics. Stars indicate significance: ** – at 1% level**

Variable	Nitrogen	P_2O_5	K_2O	Humus	Acidity
Factor 1	–0.01	–0.35	–0.18	–0.03	–0.65**
Factor 2	–0.03	0.22	–0.07	–0.03	–0.26
Factor 3	–0.01	–0.08	–0.05	–0.05	–0.30

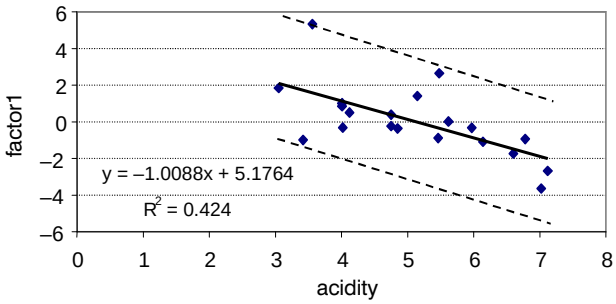

Fig. 2. Scatter plot of *Rubus idaeus* genotypes based on values from factor analysis (factor1) and soil acidity of origin site. Dash lines show the 95% confidence upper and lower limits.

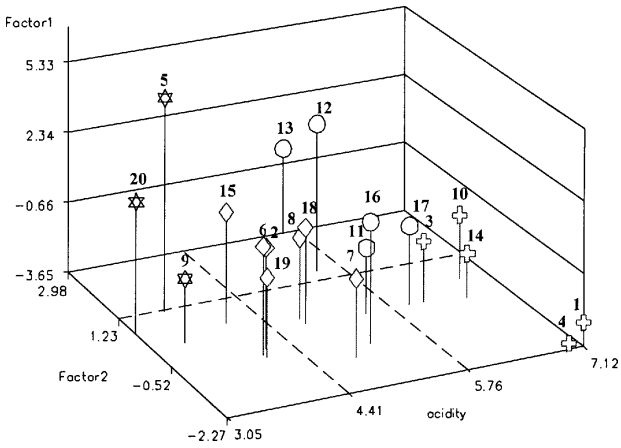

Fig. 3. Plotting of *Rubus idaeus* genotypes values in tridimensional perspective on axis of soil acidity of origin site and two axes from factor analysis of polymorphic RAPD fingerprints (SAS V8 program package). Samples are named according to DNA numbers given in Table 1. Different shapes indicate individuals grouped by soil acidity properties: star for acidity 3 to 4, diamond for 4 to 5, ball for 5 to 6, cross for 6 to 7.12

Soil acidity is a major environmental stress factor, which limits the growth of plants. In addition, acid rains also accelerate acidification of soils and cause oxidative stress [32]. Plants have evolved a complex antioxidative system in order to protect cellular membranes and organelles from the damaging effects of toxic concentrations of activated oxygen species [30, 33]. Among the primary components of this defence system are antioxidant enzymes such as superoxide dismutases, catalase and the enzymes of the ascorbate-gluthatione cycle (*e.g.*, ascorbate peroxidase, gluthatione reductase, etc.) [34]. SOD has attracted much attention in the recent years, because it is required for protecting plant cells against toxic superoxide radical induced in response to stress [35]. So, to reveal a possible genetic adaptation to soil acidity in *R. idaeus*, we studied the genetic variability in SOD isozyme profiles in various acces-

sions of this plant species. The same plants were analysed as in the RAPD study. Significant differences were detected in isozyme patterns of different accessions. Eight SOD activity bands were detected in *R. idaeus* leaf extracts. According to the number and mobility of these bands, the six main SOD enzyme profiles have been distinguished (Fig. 4). The main difference among these six SOD profiles was found in the region of gel that includes the fastest zones of SOD activity and in the second (II) SOD enzyme profile. The disappearance of isozyme 1 was detected in leaves of most plants originally grown in acidic soils (pH 3.05–4.85) (Fig. 4). However, two plants (JL 14010607 and JL 10010527), former habitants of acidic soil (Table 1), showed isozyme 1 activity. The plant (JL 05010427) that in natural habitat had grown in soil with neutral pH (pH 7.02) had the most polymorphic pattern (II) of SOD isozymes. Differences in SOD spectra could be related to the local adaptation and survival mechanism of different genotypes under natural environmental stress. It has been shown by previous studies that different stress factors (NaCl salinity, osmotic stress, heavy metals, chilling, fotodestruction) lead to different regulation of distinct SOD isozymes [30, 32, 36, 37]. It is very interesting that in some cases the disappearance of isoforms and the decline of SOD activities in leaves were detected. As Ren et al. have reported [30], the activity of one SOD isoform was greatly diminished both in leaves and roots with increasing the altitude and disappeared at a high altitude in *Plantago major*. The authors consider this SOD polymorphism in *P. major* as the adaptation of alpine plants to abiotic stress. Our results of the SOD polymorphism study are more complicated and do not show a clear adaptive dependence.

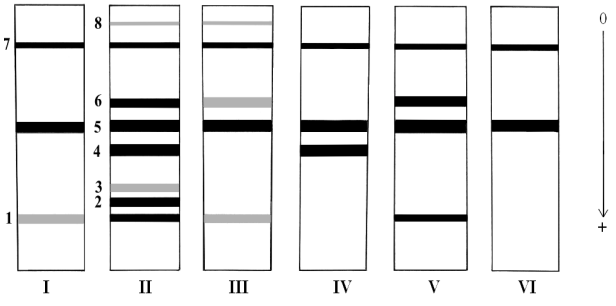

Fig. 4. Superoxide dismutase (SOD) isozyme profiles (I–VI) in *Rubus idaeus* L. leaves **I** – isozyme profile typical of the accessions JL 01010425, JL 0401027, SS 01011014, JL 06010513; **II** – JL 05010427; **III** – JL 09010523; **IV** – JL 03010425, JL 08010523, JL 11010530, JL 12010605; V – JL 14010607, JL 10010527, VI – JL 02010425, JL 15010609, JL 16010610, JL 18010625, JL 22010711, JL 36010908, JL 45010926, JL 52010930

In this situation only some DNA polymorphisms detected in the *R. idaeus* accessions studied and demonstrating a correlation with soil acidity at the site of origin can be regarded as a locality-dependent adaptive variation. Our current results, showing a certain correlation between RAPD markers and ecological factors, suggest that the former are subjected to natural selection and could be used as molecular markers of adaptive loci.

Received 12 January 2004
Accepted 17 May 2004

References

1. Hedrick PW. Trends Ecol Evol 2001; 16: 629–36.
2. Travisano M. Adaptation: Genetics Encyclopedia of Life Sciences. Nature Publishing Group, 2001: 1–6.
3. Hurme P. Genetics basis of adaptation: bud set date and frost hardiness variation in Scots pine. PhD thesis, Oulun Yliopisto, Oulu 2000; 5–16.
4. Fahima T, Sun GL, Beharav A et al. Theor Appl Genet 1999; 98: 434–47.
5. McClelland M, Welsh J. PCR Methods Appl 1994; 59–65.
6. McKay JK, Latta RS. Trends Ecol Evol 2002; 17: 285–91.
7. Volis S, Yakubov B, Shulgina I et al. Biological Journal of the Linnean Society 2001; 74: 289–303.
8. Karhu A, Hurme P, Karjalainen M et al. Theor Appl Genet 1996; 93: 215–21.
9. Williams JGK, Kubelik AR, Livak KJ et al. Nucleic Acids Res 1990; N8: 6531–35.
10. Lu MZ, Wang XR, Szmidt AE. Forest Genetics 1997; 4: 227–34.
11. Nevo E, Baum B, Beiles A et al. Genet Res Crop Evol 1998; 45: 151–9.
12. Owour ED, Fahima T, Beharav A et al. Genetica 1999; 105: 177–92.
13. Li YC, Fahima T, Beiles A et al. Theor Appl Genet 1999; 98: 873–83.
14. Semagan K, Bjornstad A, Stedje B et al. Theor Appl Genet 2000; 101: 1145–54.
15. Nevo E. Evol Biol 1988; 23: 217–46.
16. Masojć P. Cell & Mol Biol Lett 2002; 7: 499–509.
17. Graham J, Squire GR, Marshall B et al. Molecular Ecology 1997; 6: 1001–8.
18. Практикум по агрохимии. Москва, Издательство МГУ, 1989.
19. Žvingila D, Verbylaite R, Abraitis R et al. Baltic Forestry 2002; 8: 2–7.
20. Davis BJ. Ann NY Acad Sci 1964; 121: 404–27.
21. Fridovich I. Science 1978; 201: 875–80.
22. Van de Peer Y, de Wachter R. Comput Appl Biosci 1994; 10: 569–70.
23. Huang H, Dane F, Kubisia TL. American Journal of Botany 1998; 85(7): 1013–21.
24. Nei M, Li WH. Proc Natl Acad Sci USA 1979; 76: 5269–73.

25. Hufford KM, Mazer SJ. Trends Ecol Evol 2003; 18: 147–55.
26. Nevo E, Beiles A. Theor Appl Genet 1989; 77: 421–55.
27. Fahima T, Röder MS, Wendehake et al. Theor Appl Genet 2002; 104: 17–29.
28. Nevo E, Beiles A, Kaplan D et al. Evolution 1986; 40: 13–20.
29. Nevo E, Beiles A, Krugman T. Theor Appl Genet 1988; 75: 529–38.
30. Ren HX, Wang ZL, Chen X et al. Environmental and Experimental Botany 1999; 42: 51–9.
31. Lafitte HR, Champoux MC, McLaren G et al. Field Crops Research 2001; 71: 57–70.
32. Kong FX, Liu Y, Hu W et al. Chemosphere 2000; 40: 311-18.
33. Rančelis V. Biologija 1993; 4: 19–26.
34. Wang B, Lüttge U, Ratajczak R. J Plant Physiol 2003; 161: 285–93.
35. Cakmak I, Marschner H. J Exp Bot 1993; 155/156: 127–30.
36. Smirnoff N. Enviroment and Plant Metabolism: Flexibility and Acclimation. BIOS Scientific Publishers Ltd., Oxford, 1995: 217–43.
37. Cuypers A, Vangronsveld J, Clijsters H. Fre Radical Res 1999; 31: 539–43.

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MOLEKULINIŲ ŽYMENŲ PANAUDOJIMAS PAPRASTOSIOS AVIETĖS (*Rubus idaeus* L.) ADAPTYVAUS KINTAMUMO TYRIMAMS

S a n t r a u k a

Paprastosios avietės (*Rubus idaeus* L.) adaptyvus kintamumas tirtas atsitiktinai pagausintos polimorfinės DNR (angl. RAPD – *random amplified polymorphic DNA*) ir superoksismutazės (SOD) izofermentų polimorfizmo metodais. Su 36 RAPD pradmenimis tirtų pavyzdžių genominėje DNR nustatyti 284 lokusai. Iš jų 81,05% buvo polimorfiniai. Norint atskleisti koreliaciją tarp DNR polimorfizmo tirtuose augaluose ir jų pradinių augaviečių dirvos savybių, buvo išanalizuota 230 polimorfinių lokusų, panaudojus kompiuterių programų paketą SAS V8. Patikima ir pakankamai aukšta (–0,65) koreliacija nustatyta tarp dirvos, kurioje augo tirti pavyzdžiai iki patekimo į kolekciją, rūgštingumo ir RAPD žymenų polimorfizmo. Tiriant SOD izofermentų polimorfizmą tuose pačiuose *R. idaeus* pavyzdžiuose, aptikta iki aštuonių izofermentų ir šeši jų spektrų tipai. Patikimos priklausomybės tarp SOD polimorfizmo ir augaviečių dirvožemio cheminių savybių nenustatyta.